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Chromosomal Abnormalities Subdivide Ependymal Tumors into Clinically Relevant Groups

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Ependymoma occurs most frequently within the central nervous system of children and young adults. We determined relative chromosomal copy-number aberrations in 44 ependymomas using comparative genomic hybridization. The study included 24 intracranial and 20 spinal cord tumors from pediatric and adult patients. Frequent chromosomal aberrations in intracranial tumors were gain of 1q and losses on 6q, 9, and 13. Gain of 1q and loss on 9 were preferentially associated with histological grade 3 tumors. On the other hand, gain on chromosome 7 was recognized almost exclusively in spinal cord tumors, and was associated with various other chromosomal aberrations including frequent loss of 22q. We conclude that cytogenetic analysis of ependymomas may help to classify these tumors and provide leads concerning their initiation and progression. The relationship of these aberrations to patient outcome needs to be addressed. ***(Am J Pathol 2001, 158:1137–1143)***

Ependymoma is a tumor of neuroepithelial tissue that occurs in both brain and spinal cord, most frequently in children and young adults. Prognosis differs in intracranial and spinal cord tumors. Surgical resection followed by chemotherapy and/or radiotherapy are the most common treatments for intracranial tumors, but the patients frequently relapse. Overall survival and progression-free survival at 5 years are 50 to 60% and 30 to 50%, respectively.[1–3] On the contrary, recurrence is rare for ependymomas located in the spinal cord, and gross total resections using surgical microscopy do not need to be treated with adjuvant therapy.[4,5]

A prognostic marker for intracranial ependymomas would be clinically useful, and various factors have been analyzed. Several studies have identified extent of resection as an important variable.[1–9] Young age at diagnosis is a poor prognostic factor, although a clear explanation for this observation has not been proposed.[1,3,7,10,11] Ependymoma is histologically graded, and high-grade tumors are characterized by the presence of anaplasia; however, it is controversial whether grade is prognostic.[1–3,9,12–16] Tumors with higher grade have higher MIB-1 labeling index (LI), but this is not well correlated to outcome.[10,17–20]

We hypothesized that a genetic examination of tumor tissue might provide clues to tumor behavior. To date, most genetic studies of ependymomas have been based on karyotyping,[21–25] polymerase chain reaction (PCR)-based microsatellite analysis,[26–31] and NF2 sequencing.[31,32] Karyotyping depends on *in vitro* culture of surgically resected tumor tissue, and therefore may not represent the whole population of tumor cells. Microsatellite analysis provides only limited coverage over the whole genome. Comparative genomic hybridization (CGH) screens the whole genome of all cells in a tumor in a single experiment at a resolution of several megabases. Recently, a CGH study of pediatric ependymomas was published.[33] Our study adds information concerning genetic differences between intracranial and spinal cord ependymomas and between pediatric and adult cases. We determined chromosomal copy number aberrations (CNAs) of 44 ependymomas including intracranial and spinal cord tumors in pediatric and adult cases using CGH. We believe that classification of ependymomas based on their cytogenetic characteristics may help to identify a useful prognostic marker and provide clues to understanding the development of these tumors.

Materials and Methods

Tissue Samples

Samples of ependymomas from 35 patients were obtained from the Brain Tumor Research Center Tissue

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Bank at the University of California, San Francisco, six samples were from the Mayo Clinic, and three samples were from Emory University. All samples were taken from different patients. Pathological examination was performed by neuropathologists (University of California, San Francisco, cases by KA and AB, and Mayo and Emory cases by DB) according to World Health Organization criteria.[34]

Preparation and Labeling of DNA

In 26 cases, test DNA was extracted from frozen tissue of ependymomas using a standard protocol with incubation in sodium dodecyl sulfate and proteinase K followed by phenol/chloroform extraction.[35] DNA concentration was measured fluorometrically. The DNA was labeled with fluorescein isothiocyanate (FITC) by nick translation using DNA polymerase I. Reference DNA was extracted from leukocytes of normal donors and labeled with Texas Red-dUTP in the same manner. If the amount of tumor tissue was small, the DNA was amplified and labeled by degenerate oligonucleotide primer-polymerase chain reaction (DOP-PCR)[36] using FITC-dUTP (as noted below). We confirmed that DOP-PCR products from our method produced faithful CGH profiles (Hirose Y, Aldape K, Takahashi M, Berger M, Feuerstein BG, manuscript submitted).

In 17 cases, DNA was extracted from formalin-fixed and paraffin-embedded tissue section (5-μm thick) and amplified by DOP-PCR. Tissue was deparaffinized and incubated in 1× PCR Buffer (Roche, Indianapolis, IN) with 0.5% Tween-20 (Sigma Chemical Co., St. Louis, MO) and 0.4 mg/ml proteinase K (Life Technologies, Inc., Rockville, MD) for 3 days at 55°C. Proteinase K was added twice a day (1 μg per 2.5 μl of sample volume). After 3 days, proteinase K was inactivated by heating 10 to 15 minutes at 95°C, and an aliquot was subjected to DOP-PCR.

DOP-PCR amplification was performed in two phases with DNA extracts prepared as above. In the first phase (low stringency reaction), 1 μl of sample was added to the buffer containing dNTPs (dATP, dCTP, dGTP, and dTTP; Roche), DOP primer (5′-CCGACTCGAGNNNNNNATGTGG-3′, where N = A, C, G, or T) and 1′ Sequenase reaction buffer (Amersham, Cleveland, OH). The reaction was performed with five cycles at 30°C for 5 minutes, 37°C for 2 minutes, and 96°C for 2 minutes, adding Sequenase (Amersham) at each 30°C step. The first phase product was subjected to the second phase reaction where *Taq* polymerase (Roche) was used. Thermal cycle conditions were: 95°C for 5 minutes, 35 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes, followed by final extension at 72°C for 5 minutes.

DNA was labeled with another DOP-PCR reaction using digoxigenin-11-dUTP (Roche). Thermal cycle conditions were as follows: 95°C for 10 minutes, 25 cycles at 94°C for 70 seconds, 56°C for 70 seconds, and 72°C for 3 minutes, followed by final extension at 72°C for 10 minutes.

Reference DNA was amplified from 50 ng of normal male DNA and labeled as described above except that FITC-dUTP (Du Pont Inc., Wilmington, DE) was used instead of digoxigenin-dUTP.

CGH

Metaphase spreads were prepared from normal human male peripheral-blood lymphocytes stimulated with phytohemagglutinin. CGH was performed according to the procedure described by Mohapatra and colleagues.[37] The labeled DNAs were hybridized to target lymphocyte metaphase spreads. After washing, the metaphases were incubated with rhodamine-conjugated anti-digoxigenin antibody, washed, and counterstained with 4,6-diamino-2-phenylinodole in antifade solution. Red, green, and blue images were acquired with a Quantitative image processing system (QUIPS), and the ratios of fluorescence intensity along the chromosomes were quantitated.[38] A relative gain was scored when the mean test: reference ratio was >1.2 and relative loss was scored when the mean green: red ratio was <0.8.[39] CNAs were not scored at or near the centromeres. Amplifications were scored only when visual inspection revealed a bright and discrete signal confined to a subchromosomal region.

Statistical Analysis

The total numbers of CNAs for intracranial and spinal cord tumors were compared by a nonparametric Mann-Whitney test. We examined whether specific CNAs in intracranial tumors were associated with histological grade, tumor location, or type of the disease (primary or recurrent) using Fisher's exact test. Fisher's exact test was also used to test for associations among CNAs. For these analyses we considered only those CNAs that occurred in 20% or more of the total intracranial sample.

Results

Our study population consisted of 24 intracranial and 20 spinal cord tumors. Because a cursory examination of the results showed that these comprised two separate genetic groups, we analyzed the two groups separately. CGH profiles were successfully obtained from each of the 44 samples that we investigated including DNAs extracted from frozen tissue and those extracted and amplified from paraffin-embedded sections. Two cases (intracranial cases no. 10 and no. 22 in Table 1) were examined by both nick translation and DOP-PCR, and the resulting CGH profiles were similar (Figure 1; intracranial case no. 10).

Table 1 and Figure 2 are a summary of the intracranial tumors. Ten cases were diagnosed as ependymoma (World Health Organization grade 2), and 13 cases were anaplastic ependymomas (World Health Organization grade 3). Fourteen tumors were infratentorial and nine were supratentorial. CNAs were recognized in 19 (79%) of 24 tumors. CNAs frequently recognized were gains on 1q (7 cases); and losses on 6q (6 cases), 9p (9 cases), and 9q (6 cases). There were no distinguishable amplifications. The mean value of total CNAs was 2.5 per case. Correlations of these frequent CNAs to tumor histology or location are shown in Table 2. Gain on 1q occurred more frequently in grade 3 tumors, but the correlation was not

Table 1. Chromosomal Aberrations in Intracranial Ependymomas

Case	Age (yrs)	Sex	Disease	Location	Grade (WHO)	CNAs	Gain	Loss
1	0.8	M	Primary	I	3	0		
2	0.8	F	Primary	I	3	1		9p
3	2	M	Primary	S	2	1		9p
4	2	F	Recurrent	S	3	1		22q
5	2	F	Recurrent	I	2	0		
6	2	F	Recurrent	S	3	0		
7	3	M	Recurrent	I	3	2		9p, 22q
8	5	F	Primary	I	2	2	1q	10q
9	7	M	Recurrent	I	3	2	1q	9
10	9	M	Recurrent	S	3	2	1q	9
11	12	F	Primary	I	2	4		6, 10, 13q, 16
12	12	M	Recurrent	I	2	6	1q, 5pter-q21, 7q21-ter, 18	1pter-36.1, 6q
13	13	M	Primary	I	3	3		6q23-ter, 12pter-q23, 13q12-21.2
14	18	M	Primary	S	3	1		13q
15	20	F	Recurrent	S	3	6	8, 20q	3p, 9, 22q, X
16	25	M	Primary	I	2	0		
17	25	M	Primary	S	3	3	1q	9, 16q
18	27	F	Primary	I	3	8	1q, 5, 7, 9	2, 3, 6, X
19	31	M	Primary	I	2	4	12, 15q	6, X
20	33	F	Recurrent	S	3	3	1q, X	9
21	42	M	Primary	I	2	5	5, 9, 12, 15q, 19	
22	59	M	Recurrent	S	3	5		5q, 9, 11p, 13, Y
23	66	M	Primary	I	2	1		6
24	84	F	Primary	S	2	0		

I, infratentorial; S, supratentorial; CNAs, copy number aberrations.

statistically significant. Loss of 6q and loss of 9 (either p or q arm) were mutually exclusive. Cases with loss of 6q were all infratentorial tumors. Cases with loss of 9p but without loss of 9q were all intracranial and 3 years of age or younger. On the other hand, losses that included 9q were seen in six intracranial cases 7 years of age or older. This aberration correlated with histological grade 3 (P = 0.024) and supratentorial location (P = 0.050).

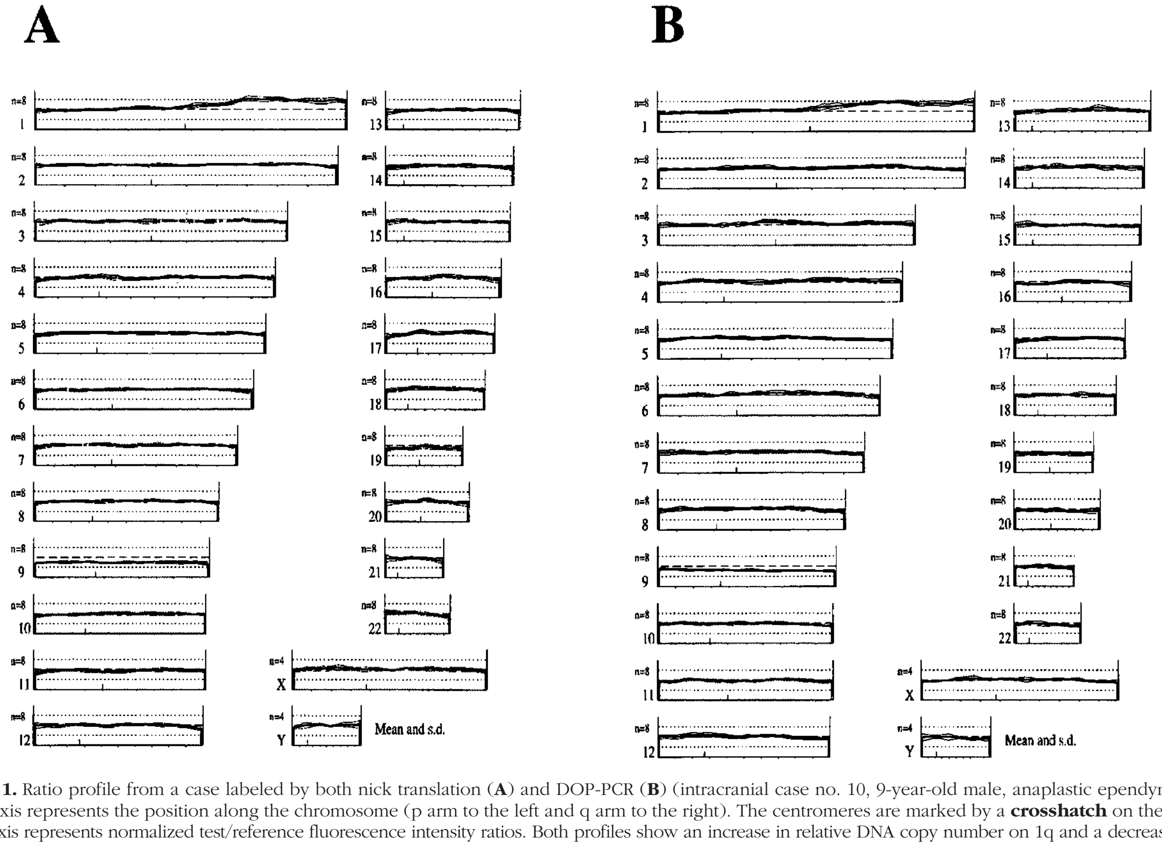

Figure 1. Ratio profile from a case labeled by both nick translation (**A**) and DOP-PCR (**B**) (intracranial case no. 10, 9-year-old male, anaplastic ependymoma). The *x* axis represents the position along the chromosome (p arm to the left and q arm to the right). The centromeres are marked by a **crosshatch** on the *x* axis. The *y* axis represents normalized test/reference fluorescence intensity ratios. Both profiles show an increase in relative DNA copy number on 1q and a decrease on 9.

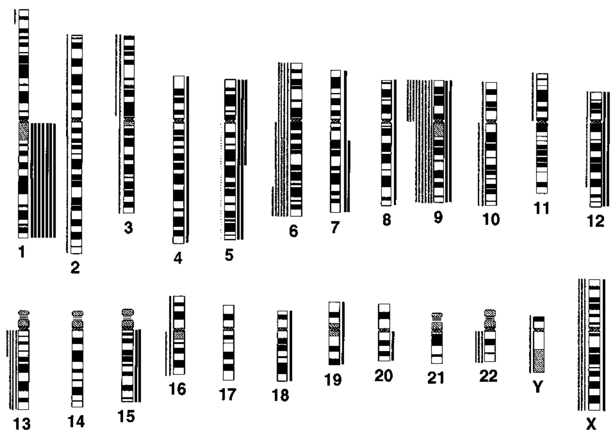

Figure 2. Summary of CNAs in 23 intracranial ependymomas. Lines to the left of each chromosome idiogram show regions of reduced relative DNA copy number, and lines to the right show regions of increased relative DNA copy number. Each line represents a CNA found in one tumor. CNAs frequently recognized in CGH for intracranial ependymomas were gain of 1q (seven cases); loss of 6 (six cases), 9p (nine cases), and 13 (four cases). There were no distinguishable amplifications.

There was no relationship between CNAs and whether disease was primary or recurrent.

Associations among these aberrations were examined (Table 3), and only association between gain on 1q and loss of 9q was significant ($P = 0.048$).

Table 4 and Figure 3 show a summary of CGH results from spinal cord tumors. All cases were primary disease. Fourteen cases were intramedullary (conventional) ependymoma (World Health Organization grade 2), and six cases were myxopapillary ependymomas. CNAs were recognized in all 20 cases. Nearly all spinal cord ependymomas had gain on chromosome 7 (19 of 20 cases). Other CNAs frequently recognized were gains on chromosome 2 (5 cases), 5 (6 cases), 9 (14 cases), 12 (8 cases), 15 (6 cases), 18 (5 cases), 20q (5 cases), and X (11 cases); and losses on 13q (4 cases) and 22q (11 cases). All aberrations involved the whole arm or the whole chromosome. There were no distinguishable amplifications.

Association among these aberrations were tested, and gain on chromosome 12 was significantly associated with gains on 15 ($P = 0.018$) and X ($P = 0.005$), and loss on 22q ($P = 0.028$). The number of total CNAs in spinal cord cases was 6.2 per case; this was significantly greater than the mean number of CNAs in intracranial cases ($P < 0.001$). Losses of chromosome 1 and 10 were recognized only in the youngest (ie, 10 years of age) spinal cord cases of myxopapillary type; loss of 22q was not recognized in myxopapillary tumors.

Table 3. Association among Frequent Aberrations in Intracranial Ependymomas

Combination	Observed cases	*P* value
+1q/−6q	2/24 (8.2%)	>0.999
+1q/−9p	4/24 (16.7%)	0.36
+1q/−9q	4/24 (16.7%)	0.038
−6q/−9p	0/24 (0.0%)	0.052
−6q/−9q	0/24 (0.0%)	0.28

Discussion

Previous studies suggested the genetic differences between intracranial and spinal cord ependymomas, however, they are focused on specific genetic locus.[31,32] Our results show clear and more remarkable cytogenetic differences between tumors that occurred in intracranial and spinal cord ependymomas. First, there were far more CNAs in spinal cord (median, 6; range, 2 to 10) than in intracranial (median, 2; range, 0 to 6) tumors. This was especially evident in the gains. Tumors of the spinal cord had a median of 4 gains (range, 0 to 8) and intracranial tumors had a median of 0 gains (range, 0 to 5). Secondly, the CNAs in these two groups were different. 19 of 20 spinal cord tumors featured gain on chromosome 7. Other frequent CNAs seen in 20% or more of the spinal cord cases included gain of 2 (5 cases), 5 (6 case), 9 (14 cases), 12 (8 cases), 15 (6 cases), 18 (5 cases), 20q (5 cases), and X (11 cases); and loss of 13q (4 cases) and 22q (11 cases); these CNAs were far less frequent in the intracranial cases. On the other hand, intracranial cases had frequent gains on 1q (7 cases) and losses on 9 (8 cases); these CNAs were nearly absent in the spinal cord tumors. This suggests that intracranial and spinal cord ependymomas progress along substantially different pathways although they comprise one histological entity. It is well known that intracranial tumors frequently relapse[1–8,10] and that spinal cord tumors rarely relapse after gross total resection.[4,5] Our data suggest that the differences in clinical behavior are related to cytogenetic profiles. Studies that compare genetic aberrations of ependymal tumors and outcome should be performed to confirm this hypothesis.

The frequency of whole chromosome 7 gain is an important difference between spinal cord and intracranial ependymomas. Only one spinal cord tumor did not have a whole gain on chromosome 7. This tumor was unusual because it had the smallest number of gains and the smallest number of CNAs among spinal cord tumors (a situation reminiscent of intracranial tumors) furthermore, it was the only spinal cord tumor with loss on 9q, a region

Table 2. Correlation of Frequent Aberrations in Intracranial Ependymomas to Tumor Grade or Location

Chromosomal aberration	Histology			Location		
	Grade 2	Grade 3	*P* value	Supratentorial	Infratentorial	*P* value
+1q	2/10 (20.0%)	5/14 (35.7%)	0.65	3/10 (30.0%)	4/14 (28.6%)	>0.999
−6q	3/10 (30.0%)	3/14 (21.4%)	0.19	0/10 (0%)	6/14 (42.9%)	0.024
−9p	1/10 (10.0%)	8/14 (57.2%)	0.033	6/10 (50.0%)	3/14 (21.4%)	0.092
−9q	0/10 (0%)	6/14 (42.9%)	0.024	5/10 (50.0%)	1/14 (7.2%)	0.05

Table 4. Chromosomal Aberrations in Spinal Cord Ependymomas*

Case	Age (years)	Sex	Location	Histology	CNAs	Gain	Loss
1	10	F	Cauda equina	MP	7	3, 7, 9, 11	1, 2, 10
2	10	M	Cauda equina	MP	4	7, 9q, 14q, 20	1, 2, 10
3	10	F	L5	E	6	5, 7, 8, 9, 18, 21	
4	12	M	Cauda equina	MP	7	7, 9, 20	2, 4, 6, 12
5	15	M	Cauda equina	MP	7	5, 7, 9, 16, 18, 20	4
6	17	M	C1-C5	E	4	7, 12, X	22q
7	17	M	C7	E	7	4, 7, 9, 12, 15q, X	22q
8	18	M	Cauda equina	MP	7	4, 7, 9, 18, 21q, X	15q
9	18	M	Cauda equina	MP	3	7, 9q, 20q	6q
10	23	M	C3	E	2		9q, 14
11	25	F	T12-L1	E	6	5, 7	1p, 13q, 14q, 22q
12	29	F	L2-L5	E	7	2, 5, 7, 9q, 17	13q, 22q
13	38	F	C3-C7	E	3	7, 9	22q
14	39	M	C5	E	4	7, 12, X	22q
15	40	F	C2-C5	E	6	2, 7, 9, 12, X	22q
16	43	M	C3-T1	E	9	2, 5, 7, 8, 9, 12, 15q, X	22q
17	44	F	C	E	8	7, 9, 15q, 18, 20, X	13, 22q
18	46	M	C7-T1	E	4	7, 12, 15q	22q
19	49	F	C5-T2	E	9	2, 7, 9, 10, 12, 15q, 18, X	4
20	51	M	C4-T2	E	10	2, 5, 7, 12, 15q, X	11, 13, 14, 22q

E, ependymoma (grade 2); MP, myxopapillary ependymoma; CNAs, copy number aberrations.
*All spinal cord cases were primary disease.

lost frequently in the intracranial group. There was only one intracranial tumor with whole gain of 7. Interestingly, this tumor had the largest number of CNAs among intracranial tumors (reminiscent of the large numbers of CNAs found in the spinal cord group). Gains on chromosome 7 are the most frequent aberration in grade 2 to 4 astrocytic tumors (Hirose Y, Aldape K, Takahashi M, Chang S, Larson D, Lamborn K, Berger M, Feuerstein BG, submitted data)[40–42] and mark radiation resistance (Kunwar S, Mohapatra G, Bollen A, Lamborn K, Prados M, Feuerstein BG, submitted data).[43] The aberrations in spinal cord ependymomas, however, differ from those in grade 2 and 3 astrocytomas. Aberrations in spinal cord ependymomas cover whole arms of chromosomes or whole chromosomes, whereas aberrations in grade 2 and 3 astrocytomas often target smaller chromosomal regions ((Hirose Y, Aldape K, Takahashi M, Chang S, Larson D, Lamborn K, Berger M, Feuerstein BG, submitted data). A similar situation occurs in neuroblastomas, where lower grade tumors have aberrations involving whole chromosomes or chromosomal arms, but higher grade tumors have aberrations that involve smaller chromosomal regions.[44]

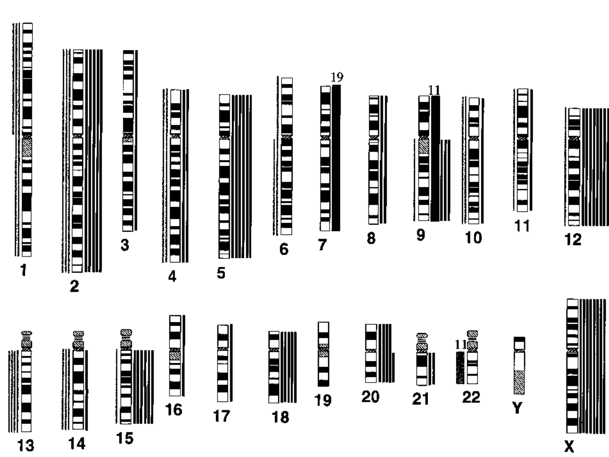

Figure 3. Summary of CNAs in 20 spinal cord ependymomas. Most cases showed gain of 7. Other frequently recognized CNAs were gain of 2 (five cases), 5 (six cases), 9 (14 cases), 12 (eight cases), 15 (six cases), 18 (five cases), 20q (five cases), and X (11 cases); loss of 2 (three cases) and 22q. The **thick lines** reflect the number of tumors (shown above the lines) with similar aberrations. There were no distinguishable amplifications.

It is believed that young patients with intracranial disease (<3 to 5 years of age) have a poorer outcome than older patients.[1,3,7,9,10] Cases 3 years of age or younger in our study had less CNAs (mean, 0.7 per case; range, 0 to 2) compared to older cases (mean, 3.3 per case; range, 0 to 8). This suggests that the clinical behavior of ependymomas may not simply be associated with the number of CNAs per case. Instead, the specific cytogenetic aberrations we see in the tumors from younger patients may be directly related to their biology. Furthermore, the only aberrations found in tumors from patients ≤3 years old were 1) loss on 9p (this lesion occurred only in young patients) and 2) loss on 22q (this lesion occurred in two of seven young patients and in 1 of 17 older patients). And three tumors from this younger group had no cytogenetic aberrations by CGH.

Thus, the cytogenetic aberrations we found in the 17 intracranial tumors from patients >3 years of age were different from those found in younger patients. These tumors had frequent gain of 1q (7cases) and losses on 6 (six cases), 9 (six cases), 13 (four cases), and X (three cases). Our pilot data suggest that cytogenetic aberrations differ in younger and older patients. These differences may underlie age-related differences in outcome.

The relationship of intracranial ependymoma grade to outcome is controversial.[1–3,9,10,13–15,20,21] Nonetheless, there were indications that gain of 1q and loss of 9 and 13 were preferentially associated with histological grade 3 (five of seven cases, six of six cases, and three of four cases, respectively) among intracranial tumors. Because *Rb* is located on 13q[45,46] and *Ink4A* is located on 9p,[47] these results suggest that the cyclin D/CDK4 pathway[48] is disrupted more frequently in grade 3 than in grade 2 intracranial ependymomas. Alterations at other members of this pathway such as cyclin D have not been described. On the other hand, although mutation of p53 is rare in ependymomas,[49,50] the p53 pathway might be altered because *Arf*, whose product stabilizes p53, is also located on 9p.[51,52] Our results are consistent with the idea that grade is associated with particular CNAs. If difficulties in grading underlie an inability to correlate grade and outcome in intracranial ependymoma, we might find that CNAs correlating with higher grade might be indicators for outcome. Further clinical studies and assays of relevant genes are needed to explore these issues.

Other interesting findings in intracranial tumors include associations of loss on 6q with infratentorial location and loss on 9q with supratentorial location (Table 2). It is possible that selection pressures in these two regions differ, resulting in different progression pathways. Such differences might affect the biology of the tumor and its response to therapy.

Loss of whole 9 was associated with gain on 1q (four of six cases), but was never seen in six cases with loss on 6q. Furthermore, loss of whole 9 and 6q were preferentially seen in supratentorial and infratentorial tumors, respectively. This suggests that there are subgroups within intracranial ependymomas characterized by chromosomal aberrations.

Our results also suggest that intramedullary spinal cord ependymomas and myxopapillary ependymomas are different genetic subgroups although both share the common genetic characteristic of chromosome 7 gain. Loss on 22q (11 tumors), gains of 15q (five tumors), and 12 (seven tumors) did not occur in myxopapillary tumors, whereas losses of chromosome 1 (two tumors), 2 (three tumors), and 10 (two tumors) occurred solely in the myxopapillary group. Loss on X (11 tumors) occurred in only one myxopapillary tumor (Table 4). It was interesting that two infratentorial intracranial tumors with gain on 12 also had gain on 15q and vice versa (Table 1). Neither of these tumors had the gain on 7 so characteristic of spinal cord tumors however. These associations suggest that a specific genetic pathway operates in myxopapillary tumors. There is also a suggestion that gains on 12 and 15q are involved in a pathway that acts both in the body of the spinal cord and in the posterior fossa. Although myxopapillary tumors grow slowly,[18] they do have a greater potential for dissemination through the central canal than other spinal ependymomas.[16] Thus, different CNAs in these two groups of spinal ependymomas may underlie differences in their clinical behavior.

Because neurofibromatosis type 2 (NF2) predisposes toward development of multiple central and peripheral nervous system tumors including ependymoma[53] and because the *NF2* gene is located on chromosome arm 22q,[54,55] studies focusing on the status of this gene in ependymomas have been performed.[26,29–32,56] These studies suggest that deletion or mutation of *NF2* is more common in spinal cord tumors than in pediatric intracranial tumors. Our data suggest that chromosome 22 is frequently lost in spinal cord ependymoma, and this is consistent with the idea that alterations of *NF2* are frequently involved in their development.

In conclusion, our pilot data suggest that intracranial and spinal cord ependymomas are different genetic diseases and comprise different subgroups within one histological entity. Furthermore, we have evidence that both intracranial and spinal cord ependymomas can be further subdivided. Categorization of these tumors by cytogenetic aberrations may help establish a classification system that predicts patient outcome. A study with larger number of cases and outcome data are needed to determine the clinical significance of the groups we have identified.

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